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Determination of Allopurinol and Oxypurinol in Rat Plasma, Intestinal Wash, and Bile by High-Performance Liquid Chromatography with Electrochemical Detection (HPLC/EC) Following Automated Solid Phase Extraction

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INTRODUCTION

Allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine), a structural analogue of hypoxanthine, is a potent xanthine oxidase inhibitor and is widely used for the treatment of gout and hyperuricemia (1,2). It was selected to demonstrate site-specific intestinal absorption in a modified *in situ* rat model developed in our laboratory (3). In the model, the rate of disappearance of the drug from intestinal lumen is monitored simultaneously with its concentrations in external jugular, hepatic, and portal venous blood and in bile. Since allopurinol is rapidly metabolized in rat blood, its major metabolite, oxypurinol (3,4-dihydroxy-3.4-d-pyrazolopyrimidine), can be used as an indicator of absorption of the parent drug. Most of the current analytical methods for the determination of allopurinol and oxypurinol have been reported for human plasma and serum. These methods involve precipitation of the proteins from plasma with various denaturing reagents including trichloroacetic, trifluoroacetic, and perchloric acids, (4,5), zinc and barium sulfate, and liquid extraction with diethyl ether/isopropanol mixture (6) as well as a direct injection of a filtered sample (7,8) onto the HPLC column.

The selectivity and sensitivity of the reported methods were found to be inadequate for the concentrations of the drug and the sample amounts typically encountered in our experiments. A method based on solid phase extraction (9) has been successfully applied to the determination of several purines including xanthine, hypoxanthine, and uric acid. Since a large number of samples is generated in each *in situ* experiment, the automated solid phase extraction procedure has been adopted for the sample cleanup. Whereas most of the reported methods used UV detection at 254 nm, both allopurinol and oxypurinol have been reported to be electrochemically active on glassy carbon electrodes (6). Electrochemical detection provides a very high selectivity and sensitivity for the determination of both allopurinol and oxypurinol. Using this technique we have developed a simple, sensitive, and robust HPLC method for the simultaneous determination of allopurinol and oxypurinol in rat plasma, intestinal wash, and bile. The method is based on a procedure utilizing automated solid phase extraction, executed entirely on the MilliLab laboratory automation system, followed by HPLC analysis with amperometric detection of allopurinol and oxypurinol in rat biological fluids.

EXPERIMENTAL

Materials

Allopurinol, oxypurinol, and 1,7-dimethyluric acid were purchased from Sigma (St. Louis, MO). Other chemicals of HPLC grade were obtained from Fluka (Ronkonkoma, NY).

Chromatography

The apparatus consisted of a Hitachi L-6000 HPLC pump, a 655-40A autosampler, a Hypersil SAS column (250 × 4.6 mm, 5 μm), and a dual LC-4B/17AT detector equipped with a glassy carbon electrode at a potential of +0.85 V (1.07 V for allopurinol in intestinal wash) *vs.* an Ag/AgCl electrode (Bioanalytical Systems, West Lafayette, IN). The mobile phase was prepared by mixing 50 ml of 1 *M* potassium dihydrogen phosphate with 50 ml of 1 *M* sodium acetate and 1.9 liters of HPLC-grade water and adjusting the pH to 6.7. The flow rate was 2.5 ml/min with the HPLC column at 50°C.

Automated Solid Phase Extraction

Solid phase extraction was performed entirely by the MilliLab LAS (Waters Associates, Milford, MA). Samples were batched in groups of 24 samples.

An aliquot (100 μl) of the internal standard solution (1 μg/ml) was added to 75 μl of rat plasma, bile, or intestinal wash (4 ml of 0.9% NaCl in water with 50 μg/ml phenol red as an unabsorbable marker, rinsed through the respective intestinal segment). The sample was allowed to stand while the system was conditioning an extraction cartridge (C-18Plus). The cartridge was conditioned with 1 ml of acetonitrile followed by 2 ml of 0.1% trifluoroacetic acid (TFA) in water. A positive air pressure was applied to force solvents through the cartridge. The sample then was loaded onto the cartridge followed by a 2.2-ml wash with 0.1% TFA in water. After the excess of solvent was removed by passing air through the cartridge for 10 sec, the analytes were selectively eluted with 0.35 ml (0.75 ml applied; 0.4 ml is retained by the sorbent) of 0.1% TFA in acetonitrile into autosampler vials. The tubes with the eluant were transferred to a Savant SpeedVac Concentrator (Savant Instruments, Farmingdale, NY) and, after evaporation (15 min at 45°C), reconstituted with 50 μl of HPLC mobile phase (see Chromatography Section). Aliquots (20 μl) of the samples were then injected onto the HPLC column.

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RESULTS AND DISCUSSION

Detection

The current–potential profile for allopurinol and oxypurinol obtained at a glassy carbon electrode detector under hydrodynamic conditions is shown in Fig. 1. The applied potential for the determination of allopurinol was set at +1.07 V. The potential above that level caused sharp increase in the background current, reducing the signal-to-noise ratio (S/N). It can be seen from Fig. 1 that oxypurinol can be detected with a very high selectivity at a potential as low as +0.85 V. Because of the improved signal-to-noise ratio, the limit of detection for oxypurinol was almost 10 times lower than that for allopurinol.

Chromatographic Separation

Both allopurinol and oxypurinol are small, slightly polar molecules which are, apparently, not ionized within the pH range 2–8. The presence of even a small concentration of organic modifiers sharply reduces their k' so that purely aqueous mobile phase is better suited for reproducible separations. The use of low concentrations (0–10%) of organic solvents with reversed-phase stationary phases often results in a poor efficiency due to the slow equilibrium across the interphase of hydrocarbon and aqueous phases. As a result, the high-carbon load reversed-phase sorbents are not highly efficient for the separation of allopurinol and oxypurinol. Their retentive behavior generally follows a mixed interaction mechanism (4). Of the different sorbents tested, a trimethyl (C1) reversed-phase sorbent SAS-Hypersil showed the best combination of retention and separation for purely aqueous mobile phase (k' = 2.05 and 2.63, α = 1.23). It also provided a highly selective elution of allopurinol and oxypurinol, minimizing the potential for interference from endogenous polar material. Representative chromatograms of allopurinol and oxypurinol in rat plasma and intestinal wash are shown in Fig. 2. All samples were spiked with the internal standard before the extraction procedure. The extracts from drug-free samples of rat plasma and intestinal wash are displayed in Figs. 2A and B, respectively. A typical chromatogram of an extracted plasma sample obtained from the hepatic vein 20 min after the administration of 2 mg/kg allopurinol via the ileal segment of the rat small intestine is shown in Fig. 2C. A chromatogram of an extracted intestinal wash sample taken from the intestinal loop at the same time is shown in Fig. 2D. The total chromatography time is 5 min, the peaks are symmetrical and well resolved, and the baseline is clean and free of interfering peaks.

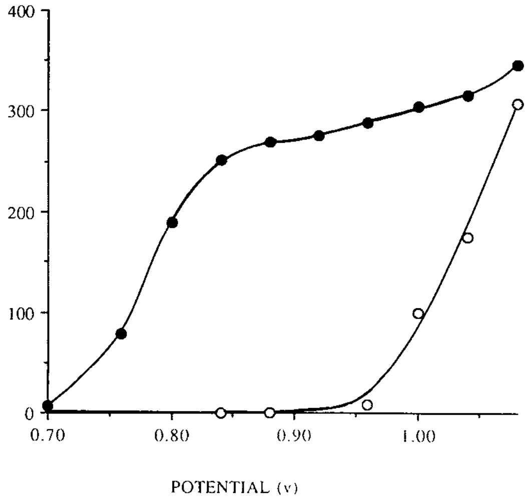

Fig. 1. Current–potential profile (hydrodynamic conditions) for oxypurinol and allopurinol (32 ng on column). (○) Allopurinol; (●) oxypurinol.

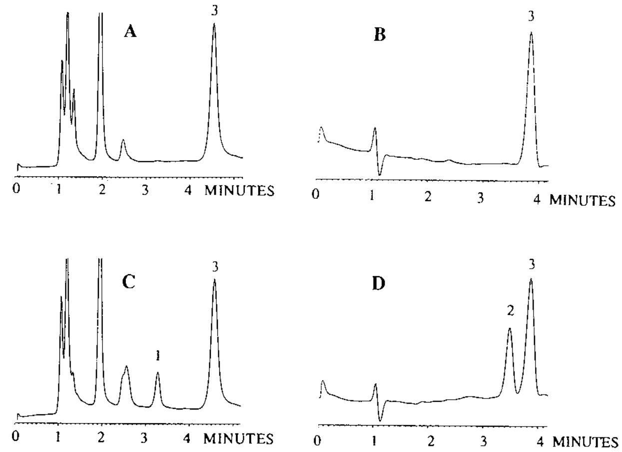

Fig. 2. Representative chromatograms of rat plasma and intestinal wash extracts spiked with 3 μg/ml internal standard, 1,7-dimethyluric acid. (A) A drug-free sample of rat plasma and (B) intestinal wash; (C) a plasma sample taken from the hepatic portal vein 20 min following administration of 2 mg/kg allopurinol via the ileal segment of rat intestine; (D) intestinal wash sample taken at the same time. (1) Oxypurinol; (2) allopurinol; (3) internal standard.

Calibration

Oxypurinol was added to drug-free rat plasma, and allopurinol to intestinal wash, to obtain respective concentrations of 1.6 μg/ml. These working standards were diluted serially to provide a standard curve for the concentration range from 6.4 to 1600 ng/ml (n = 9). Peak height ratios of drug to internal standard were plotted versus drug concentrations. The slope and intercept of the standard curve were estimated by least-squares linear regression. The calibration

Table I. Recovery Data for Allopurinol and Oxypurinol in Rat Plasma, Intestinal Wash, and Bile

Concentration	Plasma (mean ± SD) (%)	Intestinal wash (mean ± SD) (%)	Bile (mean ± SD) (%)
0.2 μg/ml oxypurinol	85.2 ± 4.1	86.3 ± 4.7	83.9 ± 6.7
1.0 μg/ml oxypurinol	88.4 ± 2.9	90.0 ± 1.4	88.8 ± 3.6
0.2 μg/ml allopurinol	88.0 ± 3.5	87.4 ± 4.6	87.3 ± 5.1
1.0 μg/ml allopurinol	89.1 ± 4.7	90.6 ± 1.9	93.7 ± 5.0

Table II. Accuracy and Precision Data for the Determination of Oxypurinol and Allopurinol in Rat Plasma and Intestinal Wash

Concentration	Day (μg/ml)	Assay (mean ± SD)	Within-day precision, % ($N = 5$)[a]	Between-day precision, % ($N = 15$)	Accuracy, %
0.1 μg/ml	1	0.097 ± 0.002			
oxypurinol	2	0.105 ± 0.001	2.71	4.54	102.0
	3	0.104 ± 0.005			
1.0 μg/ml	1	0.969 ± 0.005			
oxypurinol	2	0.981 ± 0.006	1.56	2.04	97.2
	3	0.967 ± 0.034			
0.1 μg/ml	1	0.099 ± 0.002			
allopurinol	2	0.099 ± 0.002	2.19	2.67	100.0
	3	0.102 ± 0.003			
1.0 μg/ml	1	0.960 ± 0.005			
allopurinol	2	0.973 ± 0.006	0.95	2.03	97.7
	3	0.999 ± 0.017			

[a] The within-day and between-day precision and accuracy are defined in the text.

curves were linear over the range, with the correlation coefficients exceeding 0.998 for both analytes. The detection limit for allopurinol was 0.125 ng at a potential of +1.07 V. The detection limit for oxypurinol was 31.2 pg at +0.85 V (S/N = 4).

Reproducibility and Recovery

To determine the recovery, two samples of rat plasma, intestinal wash, and bile pools were prepared at concentrations of allopurinol and oxypurinol of 0.1 and 1 μg/ml each. Replicate samples ($n = 6$) were processed as described under Experimental section and compared to concentrations of the aqueous unextracted standards (Table I). Since no significant amount of either allopurinol or oxypurinol has been detected in rat bile within an hour following allopurinol administration, the method validation was limited only to rat plasma and intestinal wash. The method was validated by performing replicate analyses ($n = 5$) of rat plasma pools spiked with oxypurinol and rat intestinal wash spiked with allopurinol (0.1 and 1.0 μg/ml) on 3 separate days. Concentrations were determined by comparison with a standard curve prepared on the day of analysis. The precision as well as the overall accuracy of the method was estimated (Table II). The within-day precision for both compounds, defined as the mean of the daily coefficients of variation at each concentration ($n = 5$), ranged from 0.9 to 2.7%. The between-day precision, expressed as the coefficient of variation of the pooled 3-day data at each concentration ($N = 15$), observed for both compounds was in the range of 2 to 4.5%. The accuracy of the method expressed as the ratio of predicted to actual concentration (C ratio) was in the range of 95 to 102%.

CONCLUSIONS

The assay is suitable for the rapid and sensitive determination of allopurinol and oxypurinol in rat bile, plasma, and intestinal wash, thus allowing accurate estimation of the rate of absorption of allopurinol from different sites of the GI tract. The technical simplicity, speed, and robustness of the method afford a reliable robotic SPE procedure, which can support the unattended analysis of 96 samples per day.

REFERENCES

1. T. F. Yu and A. B. Gutman. Effect of allopurinol (4-hydroxypyrazolo-(3,4-d)pyrimidine) on serum and urinary uric acid in primary and secondary gout. *Am. J. Med.* 37:885–891 (1964).
2. R. W. Rundles, E. N. Metz, and H. R. Silberman. Allopurinol in the treatment of gout. *Ann. Intern. Med.* 64(2):229–258 (1966).
3. P. Conzentino, E. J. Eisenberg, G. G. Liversidge, and K. C. Cundy. In situ rat model for simultaneous determination of intestinal absorption, hepatic extraction and biliary excretion. *Pharm. Res.* 6(Suppl.):113 (1989).
4. C. T. Hung, A. R. Zoest, and D. G. Perrier. Analysis of allopurinol and oxypurinol in plasma by reversed-phase HPLC. *J. Liq. Chromatogr.* 9(11):2471–2483 (1986).
5. J. M. Failler, R. Farinotti, and A. Dauphin. Quantitative liquid chromatography of allopurinol and oxypurinol in human plasma and urine. *Ther. Drug Monitor.* 7(3):324–328 (1985).
6. F. Palmisano, E. Desimoni, and P. G. Zambonin. High-performance liquid chromatography with polarographic and voltammetric anodic detection: Simultaneous determination of allopurinol, oxypurinol and uric acid in body fluids. *J. Chromatogr.* 306:205–214 (1984).
7. H. Breithaupt and G. Goebel. Determination of allopurinol and oxypurinol in biological fluids by high-performance liquid chromatography. *J. Chromatogr.* 226:237–242 (1981).
8. R. Boulieu, C. Bory, P. Baltassat, and C. Gonnet. Simultaneous determination of allopurinol, oxypurinol, hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography. *J. Chromatogr. Biomed. Appl.* 307:469–474 (1984).
9. R. A. Hartwick, D. V. Haverbeke, M. McKeag, and P. R. Brown. Sample preparation techniques prior to HPLC analysis of serum nucleosides and their bases. *J. Liq. Chromatogr.* 2(5):725–744 (1979).